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REVIEW PAPER

An Overview of Mycoviral Curing Strategies Used in Evaluating Fungal Host Fitness

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Abstract

The number of novel mycoviruses is increasing at a high pace due to advancements in sequencing technologies. As a result, an uncountable number of mycoviral sequences are available in public sequence repositories. However, only genomic information is not sufficient to understand the impact of mycoviruses on their host biology. Biological characterization is required to determine the nature of mycoviruses (cryptic, hypervirulent, or hypovirulent) and to search for mycoviruses with biocontrol and therapeutic potential. Currently, no particular selective method is used as the gold standard against these mycoviral infections. Given the importance of curing, we present an overview of procedures used in preparation of isogenic lines, along with their benefits and drawbacks. We concluded that a combination of single-spore isolation and hyphal tipping is the best fit for preparation of isogenic lines. Furthermore, recent bioinformatic approaches should be introduced in the field of mycovirology to predict virus-specific antivirals to get robust results.

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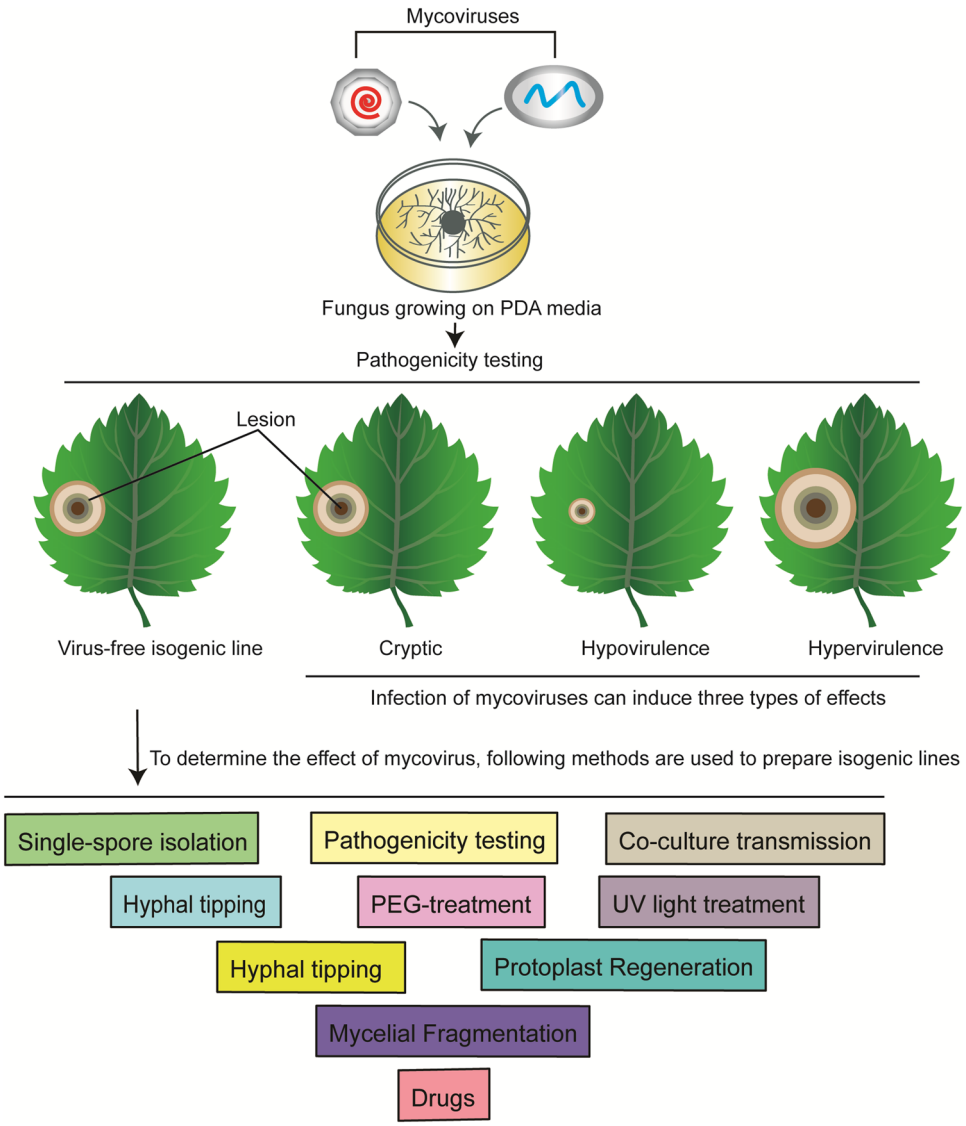
Graphical Abstract



Keywords Mycovirus · Virus-infected/free isogenic lines · Curing · Single-spore isolation · Hyphal tipping

Introduction

Mycoviruses infecting phytopathogenic fungi have attracted huge interest from researchers in the fields of virology and pathology due to their variety of impacts on host biology [1]. Nearly all of the reported fungal groups contain mycoviruses [2, 3]. These viruses are classified into 23 different families by the International Committee on Taxonomy of Viruses (ICTV) (Table 1). Mycovirus transmission is facilitated by a number of mechanisms, including hyphal anastomosis. Mycoviruses have the power to alter fungal growth and completely halt the virulence [4]. The potential of these viruses against fungal infections have previously exploited against fungal infections [5, 6]. A number of studies have shown the biocontrol potential of mycoviruses. The best example is Cryphonectria hypovirus 1 (CHV1), which has been used successfully in Europe to treat chestnut blight disease caused by *C. parasitica* [7]. This biocontrol was not commercialized due to the inability of CHV1 to infect distantly related fungal strains. A successful biocontrol agent should have the ability to transmit and replicate successfully in a distant fungal strain (by both extracellular and intracellular routes) [1]. Mycovirus-associated

Table 1 List of mycoviral families recognized by ICTV and their representative members

	Family	Genus	Representative member	Segment No.	Accession
ssDNA	*Genomoviridae*	Gemicirculavirus	Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHDV1)	1	GQ365709
		Gemytripvirus*	Gemytripvirus fungal (FgGMTV1)	3	MK430076; MK430077; MK430078
Reverse Transcribing RNA viruses	*Pseudoviridae*	Pseudovirus	Saccharomyces cerevisiae Ty1 virus (SceTy1V)	1	M18706
		Hemivirus	Saccharomyces cerevisiae Ty5 virus (SceTy5V)	1	U19263
	Metaviridae	Metavirus	Saccharomyces cerevisiae Ty3 virus (SceTy3V)	1	M34549
Double-stranded RNA viruses	*Reoviridae*	Mycoreovirus	Mycoreovirus 1 (MyRV1)	11	AY277888; AY277889; AY277890; AB179636; AB179637; AB179638; AB179639; AB179640; AB179641; AB179642; AB179643
	Totiviridae	Totivirus	Saccharomyces cerevisiae virus L-A (ScV-L-)A	1	J04692; X13426
		Victorivirus	Helminthosporium victoriae 190SV(Hv190SV)	1	U41345
	Chrysoviridae	Alphachrysovirus	Penicillium chrysogenum virus (PcV)	4	AF296439; AF296440; AF296441; AF296442
		Betachrysovirus	Botryosphaeria dothidea chrysovirus (BdCV1)	4	KF688736; KF688737; KF688738; KF688739
	Megabirnaviridae	Megabirnavirus	Rosellinia necatrix megabirnavirus 1 (RnMBV1)	2	AB512282; AB512283
	Quadriviridae	Quadrivirus	Rosellinia necatrix quadrivirus 1 (RnQV1)	4	AB620061; AB620062; AB620063; AB620064
	Partitiviridae	Alphapartitivirus	Rosellinia necatrix partitivirus 2 (RnPV2)	2	AB569997; AB569998
		Betapartitivirus	Atkinsonella hypoxylon virus (AhV)	2	L39125; L39126; L39127 (satellite)
		Gammapartitivirus	Penicillium stoloniferum virus S (PsV-S)	2	AY156521; AY156522
	Amalgaviridae	Zybavirus	Zygosaccharomyces bailii virus Z (ZbV-Z)	1	KU200450
	*Curvulaviridae**	Orthocurvulavirus*	Curvularia orthocurvulavirus 1 (CThTV)	2	EF120984; EF120985
	Polymycoviridae	Polymycovirus	Aspergillus fumigatus polymycovirus 1 (AfuTmV1)	4	HG975302; HG975303; HG975304; HG975305
		Botybirnavirus	Botrytis porri botybirnavirus 1 (BpBBV1)	2	JF716350; JF716351
	Unassigned	Unassigned	Fusarium virguiliforme dsRNA mycovirus 1 (FvV1)	1	JN671444
			Yado-nushi virus (YnV1)	1	LC061478
			Rosellinia necatrix megatotivirus 1 (RnMTV1)	1	LC333746
			Alternaria alternata virus 1 (AaV-1)	4	AB368492; AB438027; AB438028; AB438029
			Ustilaginoidea virens RNA virus M (UvRV-M)	1	KJ101567

Table 1 (continued)

	Family	Genus	Representative member	Segment No.	Accession
Positive-sense RNA viruses	*Narnaviridae*	Narnavirus	Saccharomyces 20S narnavirus (ScNV-20S)	1	M63893
	Mitoviridae	Mitovirus	Cryphonectria mitovirus 1 (CpMV1)	1	L31849
	Botourmiaviridae	Botoulivirus	Botrytis botoulivirus (BOLV)	1	LN827955
		Magoulivirus	Magnaporthe magoulivirus 1 (MOLV1)	1	LT593139
		Scleroulivirus	Sclerotinia scleroulivirus 1 (SsOLV1)	1	KP900928
		Penoulivirus	Phaeoacremonium penoulivirus (PmOLV1)	1	MK584843
		Rhizoulivirus*	Rhizoctonia rhizoulivirus (RsOLV1)	1	KP900922
	Barnaviridae	Barnavirus	Mushroom bacilliform virus (MBV)	1	MBU07551
	Hypoviridae	Hypovirus	Cryphonectria hypovirus 1 (CHV1)	1	M57938
	Endornaviridae	Alphaendornavirus	Phytophthora alphaendornavirus 1 (PEV1)	1	AJ877914
		Betaendornavirus	Sclerotinia sclerotiorum betaendornavirus 1 (SsEV1)	1	KJ123645
	Alphaflflexiviridae	Botrexvirus	Botrytis virus X (BotVX)	1	AY055762
		Sclerodarnavirus	Sclerotinia sclerotiorum debilitation-associated RNA virus (SsDRV)	1	AY147260
	Gammaflflexiviridae	Mycoflflexivirus	Botrytis virus F (BVF)	1	AF238884
	Deltaflflexiviridae	Deltaflflexivirus	Sclerotinia sclerotiorum deltaflflexivirus 1 (SsDFV1)	1	KT581451
	Unassigned	Unassigned	Diaporthe RNA virus (DRV)	1	AF142094
			Fusarium graminearum virus DK21 (FgV/DK21)	1	AY533037
			Oyster mushroom spherical virus (OMSV)	1	AY182001
			Hadaka virus 1 (HadV1)	11	LC519840; LC519841; LC519842; LC519843; LC519844; LC519845; LC519846; LC519847; LC519848; LC51984; LC519850

Table 1 (continued)

	Family	Genus	Representative member	Segment No.	Accession
Negative-sense RNA viruses	*Mymonaviridae*	Sclerotimonavirus	Sclerotinia sclerotimonavirus (SsNSRV-1)	1	KJ186782
		Botrytimonavirus*	Botrytimonavirus botrytidis (BcNSRV-7)	1	MT157413
		Lentimonavirus*	Lentinula lentmonavirus (LeNSRV-1)	1	LC466007
		Phyllomonavirus*	Phyllomonavirus phyllospherae (SLaNSRV-4)	1	KT598229
		Auricularimonavirus*	Auricularimonavirus auriculariae (AhNSRV-1)	1	MT259204
		Penicillimonavirus*	Penicillimonavirus alphapenicillii (PdNSRV-1)	1	MK584858
		Plasmopamonavirus*	Plasmopamonavirus plasmoparae (PvLAMV8)	1	MN557004
	Phenuiviridae	Lentinuvirus	Lentinula lentinuvirus (LeNSRV-2)	2	LC466008; LC466009
		Entovirus	Entoleuca entovirus (EnPLV-1)	2	MF375882; MK140653
	Unassigned	Unassigned	Fusarium poae negative-stranded RNA virus 1 (FpNSRV-1)	1	LC150618

*Taxa that are being suggested for creation or reclassification

hypovirulence has also been proposed to be used as a therapeutic against human pathogenic fungal infections [8–10]. Recent studies have reported that human pathogenic fungal strains are hosts to diverse mycoviruses [11]. The process of controlling human pathogenic fungi would be similar to that of phage therapy, where bacteriophages are used to target and cure selective bacterial infections [12]. Studies have reported that *Saccharomyces cerevisiae* (yeast) harboring mycoviruses are reported to produce killer toxins that are lethal to other sensitive fungal strains but have no influence on host fungi [13, 14]. Mycoviruses also induce interferon production in *Malassezia sympodialis* and *Penicillium stoloniferum* [15, 16]. The significant sequence similarity of Sclerotinia sclerotiorum RNA virus L (SsRV-L) to human Hepatitis E virus suggests the possibility of mycoviral replication in humans, but more research is needed in this scenario [9]. Mycovirus from *Aspergillus flavus* reproduced surface and genetic markers in the cells of patients suffering from acute lymphoblastic leukemia (ALL) disease. This character can be used as a test for the recognition of ALL patients in remission [17]. Mycovirus therapy against pulmonary aspergillosis has previously been reviewed in detail by Wendy et al. (van [18]. These properties of mycoviruses can also be utilized to enhance agricultural, industrial, and pharmaceutical applications [19].

Extraction and gel electrophoresis of dsRNA elements are one of the most widely used techniques for detecting mycoviruses [20]. The only limitation is that it can solely detect ssDNA and dsRNA mycoviruses, while those viruses that do not accumulate dsRNA replicative form can only be detected by high-throughput sequencing [21]. The second step to determine the nature of mycoviruses is biological characterization, which is an important step toward determining the impact of mycoviruses on host fitness. The effect of mycoviruses on host (fungus) biology in natural conditions is a matter of great mystery. Mycoviruses can be classified as being either hypervirulent, latent (cryptic), or hypovirulent. These terms state the changes in pathogenicity of the fungal host and do not correlate with the virulence of mycoviruses, thus creating an unclear image of mycoviruses. Cryphonectria parasitica hypovirus 1 (CHV1) infecting American chestnut (*Castanea dentata*) was reported to decrease the pathogenicity, when compared to virus-free *Cryphonectria parasitica* and was classified as a hypovirulent mycovirus [7]. Similarly, Nectria radicicola virus L1 (NrV-L1) induced greater pathogenicity in *Nectria radicicola* fungus while infecting the plants of *Panax ginseng* (ginseng) and was reported as a hypervirulent mycovirus [22]. Many mycoviruses are

cryptic or latent mycoviruses as they do not show an obvious effect on the virulence of fungal hosts [23, 24]. It cannot be said with certainty that the effect of mycoviruses on fungal biology has a direct link to the fungus pathogenicity on its respective host. For instance, the growth rate of a *Monilinia fructicola* (the fundamental agent of brown rot on *Prunus* species) was increased (in vitro) via co-infection by three mycoviruses, but there were no visible effects on its lesions growth patterns on plums when infected by its isogenic lines [25]. To effectively investigate the impact of viruses on their hosts, virus-infected and virus-free isogenic lines are required. The communal method is to eliminate the virus from infected strains or introduce the virus into virus-free fungal isolates. To date, the available literature is lacking in collective information regarding the techniques for preparing isogenic lines. The purpose of this review is to highlight the gaps and to provide a deep insight into mycoviral curing strategies to researchers in the field of fungal virology. This review covers almost all information related to the available methods used in curing mycoviral infections. Furthermore, we also describe the processes, underlying mechanisms, and their pros and cons. The information will be helpful for researchers in selecting a suitable curing method for the understudied fungal strains to evaluate the effect of mycoviruses on host biology.

Drugs Used to Treat Mycoviral Infections

A variety of antiviral drugs are developed by the scientific community to limit the spread of viruses by halting their replication [26]. An antiviral drug is a substance (small or large molecule(s), natural or synthetic) that has the ability to lessen the spread of infectious disease triggered by a virus [27]. The use of already-available drugs and antibiotics to treat mycoviral infections has been widely documented, despite the fact that no specific drugs have been specifically designed to limit mycoviral infections. Curing mycoviral infections using drugs is achieved by inoculating dsRNA-positive mycelial plugs on PDA plates containing an optimized amount of drug (varies for different fungal hosts; needs optimization, no standard available) and incubating at optimized temperature and light conditions [28]. After incubating for a few days, small marginal mycelial plugs are transferred to new PDA plates and examined for the presence of mycoviruses through RT-PCR, dsRNA, or total RNA extraction.

Cycloheximide is a naturally occurring antibiotic produced by the *Streptomyces griseus* bacterium. It blocks eukaryotic translational elongation in protein synthesis by interfering with the translocation step, i.e., the movement of mRNA and two tRNA molecules with respect to the ribosome [29]. Therefore, the primary function is to inhibit protein synthesis. Binding takes place at the ribosome and prevents eEF2-mediated translocation [30]. It is reported that the synthesis of dsRNAs in fungi is inhibited by cycloheximide treatment, which has been widely used previously. Cycloheximide prevents both the replication and translation of the viral genome from the negative-strand RNA by preventing the clearance of ribosomes from the viral genome (RNA) thus freezing ribosomes [31]. Successful elimination of mycoviruses has been reported from *Aspergillus fumigatus* [28] and *Lentinula edodes* [32]. It is also interesting to note that cycloheximide is not successful for curing all fungal strains, even at higher concentrations, e.g., *Metarhizium anisopliae* [33], *Aspergillus niger* [34], and *Pseudocercospora griseola* [35]. Partial success has been reported for *Pseudogymnoascus destructans* [36].

5′-Fluorouracil (FU) is a pyrimidine analog used in cancer chemotherapy. The basic mechanism of FU is considered to be the establishment of a covalently bound ternary complex achieved by the binding of the drugs deoxyribonucleotide (FdUMP) and N5,10-methylenetetrahydrofolate (the folate cofactor) to thymidylate synthase (TS) [37]. This inhibits the formation of thymidylate from uracil, leading to the inhibition of RNA and DNA synthesis and ultimately leading to cell death. Fluorouracil is also able to interfere with RNA processing and protein synthesis by generating a fraudulent RNA by replacing uridine triphosphate (UTP) in RNA [38]. FU can be broken down into 5-fluorouridine triphosphate, which is a substrate for viral RNA-dependent RNA polymerases. As a result, mutations are incorporated into viral genome, which are lethal for virus survival and result in loss of infectivity [39]. Ahmad et al. demonstrated that FU was ineffective against coronaviruses due to their ability to proofread. They also suggested that FU produces promising results when used in combination with deoxynucleosides [40]. The replication of Penicillium stoloniferum fast-moving virus (PsVf) was inhibited by FU treatment. FU was also successful in curing a virus from *Aspergillus flavus* [41]. FU is no longer used to prepare virus-free isogenic lines because it is ineffective against mycoviral infections.

Ribavirin (also known as tribavirin) is commonly used to treat hepatitis C, RSV infection, and some viral hemorrhagic fevers. Sometimes it is also used in combination with other drugs to get better results. Ribavirin is also reported to cure mycoviral infections. It accomplishes its antiviral activity by restraining the access of inosine monophosphate dehydrogenase (IMPDH) to its endogenous substrate, inosine-5-monophosphate through the inhibition of the enzyme [42]. This leads to a condition with minimized levels of intracellular guanosine triphosphate (GTP) pools that are required for viral replication. Studies have also indicated that the ribavirin antiviral activity might be due to inhibition of viral polymerase activity, capping of viral transcripts, or overwhelming the humoral and cellular immune responses.

Recent studies relate its primary antiviral mechanism to the deadly mutagenesis of viral RNA genomes. Being a nucleoside analog of guanosine, it also inhibits the viral polymerase [43]. A virus-free isogenic line of *Tolypocladium cylindrosporum* was prepared using ribavirin treatment [44]. *Lentinula edodes* was reported to carry a co-infection of two viruses: Lentinula edodes mycovirus HKB (LeV-HKB) and Lentinula edodes partitivirus 1 (LePV1) [44, 45]. Ribavirin treatment was only successful in curing LeV-HKB infection, while both viruses were lost during mycelial fragmentation [46]. In *Aspergillus* species, ribavirin treatment failed to eliminate mycoviruses belonging to *Fusariviridae*, *Mitoviridae*, and *Hypoviridae* [47].

Streptomycin and kanamycin have similar modes of action for viral elimination, i.e., halting protein synthesis by attaching to the ribosomal 30S subunit [48]. The infection of Sclerotinia sclerotiorum hypovirus 2 (SsHV2) and Botrytis virus F (BVF) in *Monilinia fructicola* fungus was eliminated by treatment with both of these antibiotics but was not successful in eliminating Fusarium poae virus 1 (FpV1) infection [25]. Partitiviruses are known to cause persistent infection and are difficult to eradicate [25, 49]. Rifampicin (a derivative of rifamycin) averts the attachment of RNA polymerase to DNA in order to act as an inhibitor of RNA viruses [50, 51]. Its curing mechanism is not clearly established, but in a minimal medium, a treatment of rifamycin with cAMP has been reported to cure a culture of the edible mushroom (*Pleurotus ostreatus*) from the RNA mycoviruses, i.e., oyster mushroom isometric virus (OMIV) and oyster mushroom spherical virus (OMSV) [52]. Multiple virus infections in *Ceratobasidium* sp. were eradicated using hyphal tipping and various antibiotics and drug treatments. Unexpectedly, the three mycoviruses reacted differently to each curing approach. The isolate containing Ceratobasidium endornavirus C (CbEVC) was eliminated by cycloheximide but retained by kanamycin or streptomycin. However, the isolate of CbEVD maintained stable replication with cycloheximide treatment. Interestingly, CbEVB was eliminated with all treatments [53, 54].

Single-Spore Isolation

Fungi produce an uncountable number of sexual (basidiospores and ascospores) and asexual spores (conidiophores) for propagation. Mycoviruses utilize these spores for their transmission (vertical transmission) to the next generation. Asexual (conidiophores) spores are most frequently used by mycoviruses for transmitting to progeny cells. The transmission rates vary among different mycoviruses, they range from 0% [23, 24] to 100% [55]. Single-spore isolation is commonly used to prepare virus-free and virus-infected isogenic lines for phenotype comparison and analyzing the impact of mycoviruses. Spores from the mother plate are collected and a stock spore suspension is prepared by adding them to 1 ml of sterile distilled water. The spore suspension is serially diluted as required (based on haemocytometer spore count concentration) in sterilized double-distilled water and subsequently spread on PDA plates for growth. Several single conidiophores are picked from PDA media and analyzed via classical dsRNA extraction or one-step RT-PCR for detection of mycoviruses [56–58]. It is interesting to note that the virus vertical transmission rate decreases with the age of mycelia (in most cases) [23, 24, 59, 60], but this is not true for all fungal strains [23, 24]. Successful curing has been achieved in numerous studies, few examples include *Magnaporthe oryzae* [61], *Fusarium nygamai* [23, 24], *Fusarium oxysporum* [59], *Aspergillus fumigatus* [62], *Trichoderma harzianum* [63], and edible mushrooms [64]. In some cases, single-spore isolation is reported to be unsuccessful, i.e., *Aspergillus niger* [34], *Hypomyces chrysospermus* [65], *Alternaria alternate* [57, 66–69], and *Neofusicoccum parvum* [70, 71] and in some cases, partial success has been achieved, e.g., *Diplodia seriata* [71]. Some fungal strains produce asexual structures such as pycnidia, which yield pycnidiospores (asexual spores). These are macroscopic, hard, and solid round bodies, black or dark brown in color [72, 73]. Pycnidia have a diameter of 60–200 µm and have a sub-globose shape, with the presence of an ostiole either below or bulging through the stomatal pore [73, 74]. An estimated 10,000 pycnidiospores are produced by each pycnidium, which are projected through the ostiole in a gelatinous matrix, during high humidity conditions [75]. e.g., Sclerotinia sclerotiorum strain XG36-1 was allowed to grow on sterilized carrots for 30 days in order to produce pycnidia. Harvested pycnidia were soaked in water for hydration, causing spores to be released. These spores were utilized for single-spore isolation [76]. When compared to asexual spores in the same species, vertical mycovirus transmission through sexual spores appears to be much less effective. For example, in several species of *Aspergillus*, the majority of sexual spores are virus free, whereas asexual spores of the same species are infected [77, 78]. The exclusion of dsRNA segments from sexual spores was further observed in *Ophiostoma ulmi* [79], *Gaeumannomyces graminis* strains [80], and *Helicobasidium mompa* [81]. However, in some fungal strains the transmission through sexual spores is very efficient, e.g., *Ustilago maydis* [82] and *Heterobasidion annosum* [83], *Saccharomyces cerevisiae* [84], and *Fusarium graminearum* [85].

Hyphal Tipping

The growing tips of fungi sometimes do not contain mycoviral infection, similar to the apical meristem in plants [86, 87] but this is not true for all mycoviral infections (dependent on virus replication and titer). Being cytoplasmic residents, mycoviruses are expected to move in tandem with fungal nuclei via the cytoplasmic streaming movement, as reviewed by Xiang [88]. So it is possible to get virus-free fungal strains from marginal mycelia (tips). To obtain virus-free isogenic lines, fungal strains are grown on water agar medium and incubated at the optimum temperature. Water agar is deficient in nutrients and provides a kind of stress condition by depriving fungi of nutrients, due to which fungi spread branches in search of nutrition. After 2–4 days of growth, the growing tips are dissected (~ 1 mm) under a binocular microscope and transferred to fresh water agar plates [89]. For virus-free mycelial collection, all regenerated isolates are randomly shifted to potato dextrose broth (PDB) culture and then screened for mycoviral infection. This process can be repeated 4–5 times or until virus-free cultures are obtained [53]. Alternaria alternata virus 1 (AaV-1) was cured using a mixed approach of hyphal tipping and cycloheximide treatment [90]. Hyphal tipping was not successful for *Sclerotium scleorium* and *Rhizoctonia solani* viruses [91, 92] but in most studies curing was unsuccessful [34, 93–97]. By inducing branching activities through antibiotic treatment, the number of hyphal tips should increase through rapid cell division, thus improving the chances of producing virus-free cultures from hyphal tips.

Protoplast Regeneration

The cell wall is a significant cellular structure in organisms, such as bacteria, fungi, algae, and plants. It serves a variety of purposes, including providing permeability barriers, assisting with cell design, and protecting cells in hazardous conditions [98]. The cell wall-deprived cells are termed "protoplasts" [99–101]. Similar to plant protoplasts fungal protoplasts also exhibit totipotency and can also be stimulated to produce new individuals by exposing them to appropriate external stimuli [102]. Different enzymatic methods can be applied to remove cell wall and produce protoplasts [99–101]. The cell wall of fungi are made up of polymers, i.e., dextran, mannose, chitin, and mannoproteins. Cell wall chemical composition varies from fungus to fungus and is dependent on cell development patterns and growth conditions [98]. The digestive enzymes required, such as cellulases, proteases, and chitinases, differ based on cell wall structure. As a result, the enzymatic mixture chosen from the suitable components and ratios is a critical aspect of protoplast formation. Furthermore, during cell wall breakdown, protoplasts are extremely susceptible to osmotic pressure. To maintain protoplast integrity throughout the preparation procedure, an isotonic solution of particular sugars or salts is necessary. Osmotic stabilizers are chemicals that are used to stabilize osmotic pressure, maintain cell shape, and prevent membrane breakdown.

The protoplast fusion procedure has enabled researchers to overcome the vegetative incompatibility barrier and facilitate the transmission of viruses across different fungal species/strains. For example, dsRNA from *Fusarium boothii* was transferred via protoplast fusion to other *Fusarium* species as well as the model fungus *C. parasitica* [103]. Besides this, the transfection procedure serves the purpose of introducing virus into virus-free fungal strains for host range extension, as demonstrated by Rosellinia necatrix partitivirus 1 and mycoreovirus 3 in *C. parasitica, Diaporthe sp.,* and *Valsa ceratosperma* [104]. Protoplast regeneration has been previously used to cure mycoviral infections. Virus-free isogenic lines were successfully prepared for *F. graminearum* strain HN1 using the protoplast regeneration method [105, 106]. No universal protocol is currently available as cell wall composition varies from fungus to fungus, so different digestion enzymes are required. Preparation of protoplasts (removing the cell wall) is chiefly achieved through enzymatic treatment. But physical methods like grinding and supersonic wave shocks have also been reported. However, due to practical inconvenience and the low yield of protoplasts, these methods are not widely used. A good quality protoplast can only be obtained from fresh mycelia [103]. Successful curing of mycoviral infection has been reported by [89]. An alternative way to transfer hypovirulence within a population of *C. parasitica* has been the construction of infectious cDNA clones of hypoviruses and the subsequent transgenic lines [107, 108]. An infectious clone of BVF successfully replicated in the protoplasts of *Botrytis cinerea* and maintained a stable infection [109]. CHV1 (EP713) and MyRV1 (9B21) were transferred to *Valsa mali* by fusing the protoplasts of *C. parasitica* (EP713 and 9B21) with *Valsa mali* protoplasts. The co-infection of both viruses reduced the growth and virulence of *Valsa mali* [110].

Mycelial Fragmentation

Mycelial fragmentation can be performed in a blender [111] or using screw-cap tubes and glass beads [112]. Scraped mycelia from PDA plate cultures can be transferred to bead-containing tubes, which were filled with sterile water and shaken for 10 s using a Mini-Bead Beater. Dilutions prepared from this solution can be spread on PDA plates to check for mycoviral infection using RNA extractions and

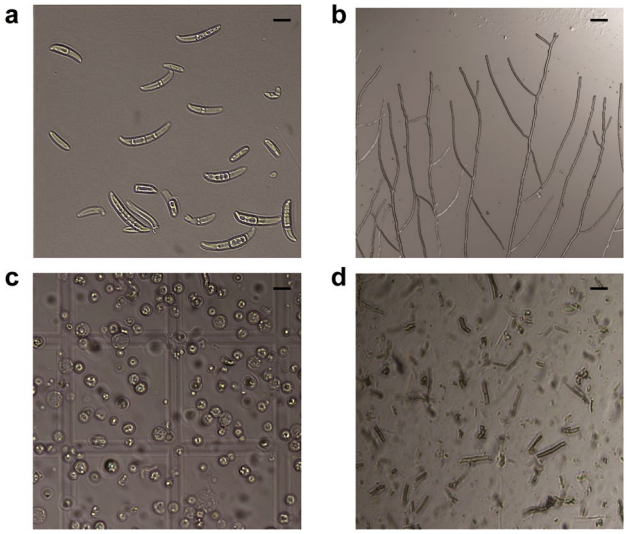


Fig. 1 A microscopic representation of **a** Spores of *Fusarium* sp. **b** Hyphal tips of *C. parasitica*. **c** Protoplast of *C. parasitica*. **d** Mycelial fragments of *Neofusicoccum parvum* (Khan et al., unpublished data)

PCR. The procedure a similar to that of single-spore isolation method. Successful curing of Pleurotus ostreatus virus (PoV) has been reported in *Pleurotus ostreatus* [113]. Prolonged incubation of mycelia results in mostly virus-free progenies. The use of mycelial fragmentation is reported in combination with drug treatment as has been reported for *Lentinula edodes* [114]. A microscopic representation of spores, hyphal tips, protoplasts, and mycelial fragments is shown in Fig. 1.

Polyethylene Glycol (PEG)-Mediated Stress

To generate water stress, water restrictors or osmotic stress inducers are widely utilized as media supplements. One of them is polyethylene glycol (PEG), which is a long-chain neutral polymer with impermeability and is soluble in water. It leads to stress in cell cultures that is similar to the drought stress seen in the cells of whole organisms [115–118]. PEG lowers the osmotic potential of the culture medium, inhibits water absorption, and is not digested by fungal strains. However, the appropriate concentrations of the inducers must be considered: the perfect concentration is one that is high enough to fulfill the experiment's goals while remaining low enough that no cultured organisms totally stop developing [119]. Polyethylene glycol (PEG) compounds are non-toxic, water-soluble polymer with different molecular weights and numerous applications [120]. PEG has been used in vivo and in vitro in different models of tissue injury and has shown many interesting biological properties; it has cytoprotective, anti-oxidant, immunosuppressive, and anti-inflammatory effects [121, 122]. PEG could create a neutralization barrier that prevents coronavirus recognition of its receptors and thus inhibiting virus entry and invasion. PEG could inhibit virus adhesion by acting as an immunocamouflage. The mode of this protection is biophysical and depends on charge massage and steric hindrance induced by the polymer. Immunocamouflage depends on molecular weight: high molecular weight PEG of 10 kDa to 35 kDa are better absorbed and consequently, they are more effective [123, 124]. It also depends on the cell surface type: small molecular weight PEGs with a molecular weight of 2 kDa were effective at binding to respiratory syncytial virus (RSV), but completely ineffective in the host cell [124].

PEG-mediated stress is reported to cure mycoviral infections, but the underlying mechanism needs to be explored. Considering the example of Pseudogymnoascus destructans, which was infected with Pseudogymnoascus destructans partitivirus-pa (PdPV-pa), whose infection, was not cured by any available methods of curing (i.e., heat therapy, nutritional and chemical stress, including cycloheximide and ribavirin, hyphal tip culture, single-spore isolation, and protoplast culture). Finally, infection was cured using PEG-induced matric potential in MM (minimal nutrition media). Different matric potential gradients were used to check for PdPV-pa infection in *P. destructans*. No visible germination of *P. destructans* mycelial growth or conidia production was observed at 5 MPa and 6 MPa. The characteristic gray pigmentation of wild-type Pd isolates turned to white in PdPV-pa free isolates after PEG treatment. The virus-free isolate also produced ominously fewer conidia than the wild-type isolate [36]. Phytophthora cactorum bunya-like viruses 1 and 2 (PcBV1 and 2) infecting *Phytophthora cactorum* were cured using PEG 8000-mediated stress [125].

Thermotherapy: Heat or Cold Treatment

Plants cultured at high temperatures (40 °C) for an extended period of time may become virus free. At higher temperatures, the activity and synthesis of virus-encoded coat proteins (CPs) and mobility proteins (MPs) are disrupted [105, 106]. When combined with the tissue culture technique, thermotherapy is more effective [126]. Apparently, the detrimental effect of high temperatures on viral proliferation and dissemination in plants reduces their concentration following heat treatment. However, viruses that require elevated temperatures for reproduction and accumulation in plant tissues increase their concentration rather than being destroyed by heat treatment, i.e., Potato spindle tuber viroid (PSTVd) [127]. They can be eliminated by meristem excision at low temperatures (between 5 °C and 10 °C). The heat tolerance of the host and the type of virus determine the duration and intensity of thermotherapy [87]. For viral eradication in potatoes, meristem culture combined with thermotherapy is often utilized. This process is completed

by incubating virus-infected plants in a growth chamber for 2–6 weeks at a light intensity of 30–50 mol m/2 s/1 and a temperature of 35 °C–37 °C. Following the corresponding duration of thermotherapy, the meristems are removed and grown on nutritional medium for regeneration [128, 129]. Virus elimination has also observed when fungal samples are stored at lowered temperatures or when stored in glycerol stocks at − 80 °C [25].

Malassezia sympodialis harboring Malassezia sympodialis mycovirus 1 (MsMV1) was cured by exposing it to high temperatures (37 °C). At this temperature, virus replication and persistence may interfere with important biological functions. Furthermore, high temperatures are thought to increase heat shocks and misfolded proteins in the cell [130], leading to virus loss owing to overworked Hsp90 if complete Hsp90 activity is necessary for viral replication [15]. Golubev et al. failed to cure *Alternaria alternata* partitivirus 1 (AtPV1) infection using a compound approach of thermotherapy, chemotherapy, spheroplast regeneration, and PEG-induced matric stress [131]. The same was observed for Sclerotinia sclerotiorum mitovirus 1 (SsMV1/KL-1) and SsMV2/KL-1 infecting *S. sclerotiorum* [132].

UltraViolet (UV) Light Exposure

Viruses are more vulnerable to UV radiation than red and white blood cells because they absorb significantly more energy. The killed viral fragments elicit a vaccination-like response, therefore boosting the immune system and producing resistance to the specific pathogen. UV radiation has a profound impact on airborne viruses. Far-UV radiation has the ability to destroy viruses. [133]. UV light has also been used to sterilize food items. The use of UV light for airborne disinfection was first shown more than eight decades ago [134]. Drug-sensitive, multidrug-resistant bacteria and different strains of viruses are inactivated by germicidal UV light [135]. Water is disinfected using germicidal lamps and UV radiation with a wavelength of 240 to 280 nm. Spectroscopy is often used to measure the average amounts of nucleic acids (DNA or RNA) present in a mixture, as well as their purity; the peak of DNA and RNA absorption is at 260 nm. UV germicidal activity is a well-known method for inactivating or killing microorganisms by causing nucleic acid damage. UV radiation with a wavelength of 300 to 320 nm is employed for light treatment in medical applications, e.g., 311 nm [136]. Ultraviolet B (UVB) narrowband lamps are used in phototherapy to treat T-cell cutaneous lymphoma and psoriasis, such as mycosis fungoides [137]. UV radiations have not been widely reported to be used for curing mycoviral infections, but a partial success was obtained by Castillo and colleagues [138]. It is not a widely used technique for eliminating mycoviruses as increasing UV light exposure may also kill fungi.

Co-Culture Transmission

Mycoviruses with persistent infections (from the families *Partitiviridae* and *Endornaviridae*) are difficult to eradicate using current curing methods. However, they can be transmitted to other compatible fungal strains to prepare virus-free and infected isogenic lines for evaluating their effect on host fitness. The dual-culture or co-culture technique is mostly used to transfer viruses to compatible fungal strains [128, 129, 139, 140]. However, depending on virus infectivity and host defense mechanisms, viruses may not be transferred to compatible strains [68, 131]. The virus-infected strain acts as a donor, while the other strain functions as a recipient. Both fungal strains are cultured side by side on the same PDA plate and allowed to grow under optimum conditions. After successful fusion of hyphae, three locations on the recipient side (i.e., near, far, and middle regions) are analyzed for the detection of mycovirus transmission. One-step RT-PCR or dsRNA extraction can be performed to check for the successful transmission of mycoviruses. The transmission of mycoviruses is hindered by vegetative incompatibility (VIC) among distant fungal strains. Allorecognition, or non-self-recognition, is a common phenomenon in the fungal kingdom, allowing them to distinguish one another [141]. This system is thought to have evolved to limit the spread of harmful organisms, like mycoviruses [142]. In many model organisms, this vegetative/heterokaryon incompatibility system is under the regulation of multiple allelic or non-allelic *vic* or *het* genes and most species seem to involve different genes [141, 143]. Non-self-recognition leads to compartmentalization between two isolates of dissimilar mycelial compatibility, leading to programmed cell death in order to interrupt fusion between hyphae and is known as heterokaryon incompatibility [144]. The mycovirus transmission between *Aspergillus* and *Cryphonectria* was unsuccessful [77, 145]. A recently reported virus, Cryphonectria naterciae fusagravirus (CnFGV1) infecting *Cryphonectria naterciae* was reported to cross vegetative compatibility barrier and was transferred to *Cryphonectria carpinicola* and *Cryphonectria radicalis* during co-culture [146]. Zinc chloride is previously reported to suppress the incompatible reaction (VIC) between two fungal strains and increase hyphal anastomosis, which can help in successful transmission of mycoviruses [147]. Zinc ions are also known to inhibit apoptosis by targeting caspase-3 activation in mammalian cells [148]. Some mycoviruses are found to be responsible for suppressing the fungal host's non-self-recognition, enabling the heterologous transmission of mycoviruses. Sclerotinia sclerotiorum mycoreovirus 4 (SsMYRV4), associated with hypovirulence in *Sclerotinia sclerotiorum*, has been reported to suppress non-self-recognition of the host as well as enable viral co-infection across vegetative

incompatible groups, through horizontal transmission of mycoviruses [149]. Vegetative incompatibility genes (*het* or *vic*) and proteins (heterotrimeric G proteins) are inhibited by SsMYRV4 [149]. In vitro horizontal transmission of mycoviruses is a commonly used technique in mycovirology. Carbone et al. found that transmission may occur in one direction, while it may not be as efficient in the reciprocal pairing [150].

Fungal Phenotyping and Pathogenicity Testing

After obtaining virus-infected and virus-free isogenic lines, fungal strains are evaluated by phenotype comparison and pathogenicity testing. Changes in colony diameter, pigmentation, and sporulation are usually assessed for determining the nature of mycovirus. The pros and cons of available methods are mentioned in Table 2. Mostly conidial suspension or mycelial plugs are used for pathogenicity testing [151, 152]. The lesion diameters of virus-infected and virus-free fungal isolates are compared in order to determine the impact of mycovirus on the pathogenicity of its host. There is a complex correlation between the occurrence of mycovirus and its effects on the fungal hosts, but overall, these effects are categorized as hypovirulence, hypervirulence, and cryptic. It is still unclear how mycoviruses regulate hosts biology [153]. In the case of a single infection, assessing the effect of mycovirus is very simple but in multiple infections, it is very difficult to link a particular phenotype with understudied mycoviruses.

Challenges in Curing Mycoviral Infections

After curing attempts, mycoviruses can be found differently distributed in the mycelia due to which they are not detected with one-step RT-PCR and can reappear after subculturing or when antibiotic stress is removed. Mycoviruses have previously been reported to reappear in various fungal strains. The titer of a 12-kb dsRNA virus in *Rhizoctonia solani* went below the level of detection by RT-PCR using hyphal tipping and extended incubation on cycloheximide media. But the virus was detected again after subculturing [154]. In another case, mycovirus was detected again in a previously reported cured isolate [154, 155]. The infection of Agaricus bisporus endornavirus 1 (AbEV1) was cured with hygromycin B, but recovered after one month when the antibiotic stress was removed [156]. Similarly, a co-infection of two mycoviruses in

Table 2 Pros and Cons of techniques available for curing mycoviral infections

Technique	Pros	Cons
Drugs/Antibiotics	Easy to use High success rate when virus-specific drugs are used	Development of resistance Least focused in mycovirology Some drugs may kill fungal strain
Single-spore Isolation	High success rate Easy to use Not expensive Not harmful for fungal strain	Time consuming Cannot be used for fungal strains which do not produce asexual spores on growth media
Hyphal Tipping	Mostly used for those fungal strains which don't produce asexual spores on growth media Not expensive Easy to use	Time consuming
Protoplast Regeneration	cDNA infectious clones of mycovirus can easily infect fungal protoplast Protoplast fusion, sometimes produce virus-free fungal strain	Sensitive process, require careful handling Low success rate Required chemicals could be expensive
Polyethylene Glycol (PEG)	Easy to use	Low success rate Not widely used against mycoviruses
Thermotherapy	Easy to use Not expensive	Exposure to high temperatures may kill fungal strains
UltraViolet Light	Easy to use	Long exposure duration may kill fungal strain and small duration may be ineffective in curing Needs optimization
Mycelial Fragmentation	Is mostly used for those fungal strains which don't produce asexual spores on growth media	Time consuming Low success rate
Co-culture Transmission	Successful in transferring mycovirus to compatible fungal groups	Mycovirus cannot be transferred to compatible fungal strains

Pseudogymnoascus destructans was cured using cycloheximide and ribavirin, but they reappeared after removal of antibiotic stress [36]. Although it was hypothesized that the use of antibiotics reduced the concentration of dsRNA below the threshold for RT-PCR detection, no quantitative real-time qRT-PCR assays were conducted to assess the viral titers before and after treatments. The existence of integrated DNA copies of the dsRNA components might be a less reasonable explanation for the apparent reintroduction of dsRNA elements from fungus [154]. This argument is supported by the indication of sequence identity between *R. solani's* dsRNA segments and portions of its genome [157]. More proof for this conceivable explanation would come from an analysis of the alignment between the mycovirus genome sequences and the fungal host genome.

The second problem is vegetative incompatibility, which we already discussed in detail. Some mycoviruses can successfully transfect protoplasts of distant fungal strains but are knocked out after subsequent subculturing. Here, we consider the example of Botrytis gemydayirivirus 1 (BGDaV1), whose virions successfully infected the protoplasts of *Botrytis cinerea* but were lost after the third subculture [158]. Neofusicoccum parvum victorivirus 3 (NpVV3) virions also successfully infected Δ*dcl2* mutant strain of *C. parasitica* but was lost after repeated subculturing [70, 71]. Similar results were observed for Rosellinia necatrix victorivirus 1 (RnVV1) [93]. These results suggested that host RNA silencing works as a counter defense against these viruses [159].

Conclusions

Mycoviruses are the most neglected group in the fields of virology and pathology, due to which our knowledge of global viral diversity in nature is still very incomplete and biased. Nevertheless, there has been a significant advancement in the taxonomy of mycoviruses based on genomic sequences and biological traits, including host antiviral responses. Recent studies have also highlighted the significance of mycoviruses as therapeutic and preventive agents against human and phytopathogenic fungal infections. Considering the importance of mycoviruses, it is very important to investigate the effects of mycoviruses that they induce in their hosts. Preparing virus-free and infected isogenic lines is the first step toward determining the nature of mycoviruses. Secondly, this process also results in the identification of mycoviruses with biocontrol potential. Previously, several methods were used to obtain virus-free isogenic lines of fungal strains. Cycloheximide and Ribavirin are among the most commonly used drugs against mycoviruses. Unfortunately, mycoviruses are developing resistance against available drugs. The unavailability of mycovirus-specific drugs is one of the limiting factors in biological characterization. Thermotherapy, UV treatment, and PEG-induced matric stress are all considered primitive techniques that are now rarely used and not very effective. Single-spore isolation, hyphal tipping, and mycelial fragmentation are among the most frequently used techniques and are considered more effective compared to other available techniques. Compared to drug treatment, the later techniques are more time consuming and labor intensive. The majority of studies revealed that combining these techniques yielded more effective results. Co-culture transmission assays are used to transfer viruses (with persistent infections) to other virus free fungal strains to investigate their impact on host biology by comparing the colony diameter. Pathogenicity tests are also performed to assess the pathogenicity of fungal strains that have and have not been infected with a virus. The size of the lesion will determine the cryptic hypo- and hypervirulent effects.

Future Directions

Recent advanced bioinformatics approaches, including molecular docking and simulation studies, should be introduced to design potential ligands or drugs to inhibit the replication of mycoviruses specifically by binding to their RNA-dependent RNA polymerase (RdRP), which is commonly present in all mycoviruses. Furthermore, suppressors of vegetative incompatibility genes (VIC) can also be designed, which will allow researchers to study the effect of viruses on a wide range of hosts. The field of nanotechnology can also be utilized for curing mycoviral infections using chemically coated nanoparticles that have antiviral activity and can specifically target mycoviruses. Polyethylene glycol-coated zinc oxide nanoparticles are already used as an effective nanoweapon to fight against herpes simplex virus type 1 [160]. Based on these suggestions, we will achieve a broader picture of viral cross-species transmission, which should in turn inform viral emergence studies. Progression in the field of mycovirology will lead to a tremendous change and soon mycoviruses will be used as bioweapons against fungal infections.

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